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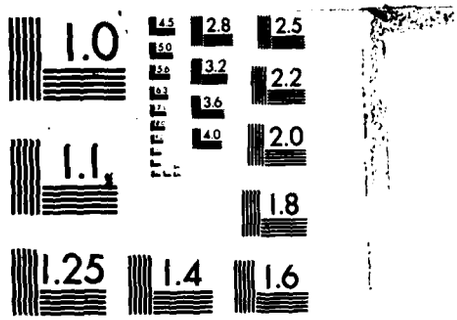

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THE MANUFACTURE AND STUDY OF HEMOGLOBIN-SALINE SOLUTION

Final Report

Gerald S. Moss, M.D.

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FINAL SCIENTIFIC REPORT: DAMD17-78-C-8043

STUDIES COMPLETED DURING THE TERM OF THIS CONTRACT WILL BE SUMMARIZED IN ABSTRACT FORM

1. SENSITIVITY OF THE IL 282 CO-OXIMETER TO LOW HEMOGLOBIN CONCENTRATION AND HIGH PROPORTIONS OF METHEMOGLOBIN

Studies on use of stroma-free hemoglobin solution as a resuscitative fluid necessitated the measurements of the chemical characteristics of the solution during total-exchange transfusion in baboons. These included the monitoring of plasma and urine hemoglobin and methemoglobin concentrations. The IL 282 Co-oximeter (Instrumentation Laboratory, Inc., Bedford, MA 02173), in principle, provides a simple means of monitoring these variables. During the course of the exchange, hemoglobin concentrations were below the stated sensitivity of the instrument (60 g/L), and the methemoglobin content exceeded the net percentages specified (10% of total hemoglobin). Thus we needed to assess the performance of the instrument beyond the specifications of the manufacturer.

We obtained 29 samples (17 plasma and 12 urine) from baboons during total-exchange transfusion with stroma-free hemoglobin solution. Plasma hemoglobin concentrations ranged from 7 to 73 g/L. All samples were assayed with the IL 282 Co-oximeter for total hemoglobin concentrations. The samples were also analyzed by the cyanmethemoglobin method with the Harleco hemoglobin kit (1).

We also assayed 13 plasma samples for methemoglobin by the Michael and Harris method (2) and with the Il 282 Co-oximeter. The range of methemoglobin values studied was 7 to 53% of total hemoglobin.

A plot of hemoglobin values obtained with the IL 282 (y) vs those measured by the cyanmethemoglobin method (x) gave a straight line by the method of least squares. The linear correlation coefficient was 0.989, which was significant ($p<0.001$). The slope of the line was 1.02, the intercept 0.16. Similarly, the correlation between methemoglobin values obtained from the IL 282 (y) and those measured by the comparison method (x) was linear. The linear correlation coefficient was 0.984, which was significant ($p<0.001$). The slope of the line was 0.982, the intercept 3.02.

The IL 282 Co-oximeter has been shown (3) to provide reproducible and accurate values for total hemoglobin, and correlates significantly with the comparison method for hemoglobin concentrations ranging from 23 to 240 g/L. No data on the methemoglobin concentrations beyond the specifications of the manufacturer (10% methemoglobin) are available. Our data show that for hemoglobin concentrations as low as 7 g/L, the correlation with the cyanmethemoglobin method is highly significant. The same is true for methemoglobin, when compared with results by the Drabkin/Austin method.

Considering the simplicity of operation of the instrument, the obviation of reagent preparations and calculations, and the range and accuracy of the data generated, the IL 282 Co-oximeter appears to be the instrument of choice for total hemoglobin and percent methemoglobin determinations over a very wide range of concentrations.

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2. PERFORMANCE OF THE OXYGEN-HEMOGLOBIN DISSOCIATION ANALYZER ("HEM-O-SCAN"), COMPARED WITH THE IL 282 CO-OXIMETER

Studies on the use of stroma-free hemoglobin solution as a temporary erythrocyte substitute necessitated the measurement of the oxygen-hemoglobin dissociation curve on samples of whole blood obtained from baboons during exchange transfusion (1). With the "Hem-O-Scan" oxygen dissociation analyzer (American Instrument Co., Silver Spring, MD 20910) this curve, in which percent saturation of hemoglobin with oxygen is the y-axis and the partial pressure of oxygen (PO_2) is the x-axis, can be simply and easily obtained. The manufacturer specifies the reproducibility of P_{50} (the PO_2 at 50% saturation) determinations for human whole blood or hemolysate, but no data are available concerning the accuracy of the P_{50} value, or of any other point on the dissociation curve. We assessed the accuracy of the Hem-O-Scan under our experimental conditions.

We obtained anaerobically 62 samples of mixed venous blood from 12 baboons that were undergoing extensive exchange transfusion with stroma-free hemoglobin solutions. Aliquots of each sample were analyzed with a IL 813 blood-gas analyzer (Instrumentation Laboratory, Inc., Lexington, MA 02173) for pH, pCO_2, and PO_2; with an IL 282 Co-oximeter for oxygen saturation, total hemoglobin, methemoglobin, and carboxyhemoglobin; with a Damon IEC MB centrifuge for hematocrit; and with the Hem-O-Scan for a dissociation curve.

The saturation, as measured with the Co-oximeter, was corrected for the proportion of methemoglobin and carboxyhemoglobin, to reflect the percentage of functional hemoglobin that in fact was oxygenated. This corrected saturation was accepted as the true value (2,3). The oxygen saturation of each sample was evaluated from the corresponding dissociation curve, by reading the ordinate of the curve at the value on the abscissa corresponding to the mixed venous PO_2.

Hematocrits of the 62 samples ranged from 0.1 to 42.0%, total hemoglobin concentrations from 36 to 141 g/L, and methemoglobin values from 0.0 to 21.6%. The mixed venous PO_2's ranged from 0.133 to 8.2 kPa (4.0 to 61.7 mmHg). The corrected percentage saturations, as determined from the Co-oximeter, varied from 26 to 87%.

A plot of the 62 saturation data obtained from the Hem-O-Scan dissociation curve (y) vs those measured from the Co-oximeter (x) was linear by the method of least squares. The linear correlation coefficient was 0.907, which was significant ($p<0.001$). The slope of the line was 1.094, the intercept -5.7%.

These results show that the Hem-O-Scan provides accurate information concerning the relationship between PO_2 and oxygen-hemoglobin saturation over almost the entire range of the dissociation curve. Such accuracy is required to obtain information about a single point on the curve such as the P_{50}; or the shape of the curve, as reflected in the Hill coefficient; or changes in the position of the curve, such as measured by the Bohr and Haldane coefficients (4). Furthermore, we found the Hem-O-Scan to be accurate over a wide range of hematocrits, hemoglobin concentrations, and proportions of methemoglobin.

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3. DETERMINATION OF SERUM CREATININE IN THE PRESENCE OF HIGH CONCENTRATIONS OF FREE HEMOGLOBIN

In studying the use of stroma-free hemoglobin solution as a temporary erythrocyte substitute we needed to monitor organ function during exchange transfusion in adult baboons. Creatinine clearance was of special concern because transient changes in renal function have been reported after infusion of a stroma-free hemoglobin solution (1). Our serum samples contained significant amounts of free hemoglobin. Thus we tested the feasibility of using a modification of the direct creatinine procedure of Heinegard and Tinderstrom (2). Their method involves a protein-solubilizing agent that acts upon serum protein, eliminating the need for protein precipitation. Inhibitors and buffers have been added to limit the action of interfering substances.

We collected 46 serum samples from eight adult baboons before, during, and after they were exchange-transfused with an 80 g/L solution of stroma-free hemoglobin.

The hemoglobin concentration of these serum samples, as determined with the IL 282 Co-Oximeter (3), ranged from 0 to 40 g/L. Creatinine was determined in protein-free filtrate by the method of Owens et al. (4), which is based on the Jaffe reaction (5). Parallel determinations on the same sera, untreated, were done by the direct method with a kit (DMA Medical Associates, Inc., Arlington, TX 76011) based on a modification of the colorimetric method of Heinegard and Tinderstrom (2). We modified the DMA method by including a test blank and by adding the sample to the test blank tube just before reading the absorbance. The creatinine concentrations of the samples as determined by the method of Owens et al. (4) ranged from 3 to 41 mg/L and were linearly related to values by our modified DMA method. The linear correlation coefficient was 0.994, the slope of the line was 0.99, and the intercept was 0.022.

A plot of the difference between the values obtained by the two methods (y) vs the hemoglobin concentration (x) gave a straight line (slope -0.008, intercept 0.006) without significant correlation. This indicates that our modified DMA method is independent of hemoglobin concentration.

Our data indicate that the direct creatinine procedure can be used with significantly hemolyzed samples when appropriate test blanks are used. The suggested method of blanking may be applicable to other assays as well. This finding is of special importance in pediatric cases, where hemolyzed samples are more common, because it may diminish requests for repeat samples.

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4. LARGE VOLUME PREPARATION OF PYRIDOXALATED STROMA-FREE HEMOGLOBIN WITH HIGH IN VIVO P_{50}

Introduction

The purpose of this paper is to describe a simple and rapid technique for the preparation of large quantities of concentrated pyridoxalated hemoglobin (PLP-Hb).

Materials and Methods

Washed outdated cells (5-10 liters) are hemolyzed by pressure shock lysis under 1000 psi nitrogen pressure. The stroma is removed by acid precipitation at a pH of 5.7 and subsequent high speed centrifugation (48,000 g). The supernatant is readjusted to a pH of 7.4 and passed

through a series of filtration cartridges from 1.2μ to 0.22 μ pore size. Organic ligands ions are removed from the solution by hollow fiber ultrafiltration against deionized water. The stroma-free hemoglobin solution (18-20 gm%) is adjusted to a pH of 7.40 with 0.1M Tris and PLP is added on a 4:1 molar ratio to hemoglobin. The solution is deoxygenated with nitrogen using a bubble gas exchanger, and the PLP co-valently bound to hemoglobin with $NaBH_4$ under nitrogen. Excess $NaBH_4$ and PLP are removed by ultrafiltration. Dilution to the desired hemoglobin concentration and electrolyte balance is made with renal dialysis concentrate. The solution is sterilized by passage through a 0.22 μ filter into transfer packs.

In vivo P_{50} of plasma Hb is obtained from baboons after exchange transfusion of the PLP-Hb solution.

Results

The characteristics of the PLP-Hb solution are given in the table. The P_{50} has been adjusted to standard conditions (pH = 7.40, PCO_2 =40 mmHg, Temp ==37°C).

Solution Characteristics	Range of Values
Yield (Hb) mass	85-90% of lysate
Volume	16-32 liters
Hemoglobin	6.5-8.0 gm%
Methemoglobin	3.0-7.0% of total Hb
Non-Hb protein	0.6-0.8 gm%
Colloid oncotic pressure	20-25 mmHg
2,3-DPG	1.5-3.0 μ M/gmHb
In vivo P50 (plasma Hb)	22.0-26.0 mmHg
Hill Coefficient	1.6-2.5
Bohr Coefficient	-0.3 to -0.2
Phospholipids	5.0-10.0% of lysate
Coagulation (P.T., A.P.T.T.)	normal

Conclusion

A simple technique for large volume preparation of PLP-Hb is described that results in a hemoglobin solution with decreased in vivo oxygen affinity.

5. OXYGEN TRANSPORT BY PYRIDOXALATED STROMA FREE HEMOGLOBIN

We have previously studied oxygen transport in baboons rendered free of erythrocytes by total exchange transfusion with stroma free hemoglobin (SFH). Although oxygen consumption, cardiac output, and arteriovenous oxygen content difference are maintained at baseline values, a significant decrease occurs in the mixed venous pO_2 to compensate for the high oxygen affinity of SFH (P_{50} = 12 mm.Hg.). Pyridoxalat hemoglobin (SFH-P) has a significantly reduced oxygen affinity (P_{50} = mmHg) compared to SFH. The object of this report is to compare the o> n dynamics in baboons exchange transfused with either SFH or SFH-P.

Eight awake, paralyzed baboons were randomly assigned to an SFH or SFH-P group. A total exchange transfusion was performed, and measurements obtained at hematocrits of 20, 10, 5, 2, and 0.

Oxygen consumption, cardiac output, and arteriovenous oxygen content difference remained constant, and were not different with SFH or SFH-P. The principle difference was a significantly higher mixed venous pO_2 in the SFH-P animals at all hematocrits below 20. Table I lists the mean ± SEM of the mixed venous pO_2 values at each hematocrit.

TABLE I Mixed Venous pO_2 (mm.Hg.)

	Baseline	20	10*	5*	<5*
SFH	49.6±3.4	34.0±6.2	22.4±3.6	13.8±2.4	13.6±2.7
SFH-P	52.3±3.9	42.2±5.5	40.7±3.4	31.3±3.0	22.0±2.5

* Difference significant, P<.05

Conclusion: The results demonstrate that SFH-P permits unloading of oxygen at higher tissue oxygen tensions than SFH, and is therefore more physiologically suited to minimize tissue hypoxia.

6. CARDIAC RESPONSE DURING HEMODILUTION WITH STROMA-FREE HEMOGLOBIN SOLUTIONS HAVING DIFFERENT OXYGEN AFFINITIES

We have shown previously that normovolemic anemia produced by exchange transfusion with Dextran-70 causes a linear increase in cardiac output, while a similar exchange transfusion with a stroma-free hemoglobin solution (SFH) produces no such change in the awake baboon. Oxygen consumption was unchanged in both groups (1). The P_{50} was 14 mmHg in the SFH group, at the lowest hematocrits. It is not clear whether the unchanged cardiac output was due to myocardial hypoxia, related to the high O_2-affinity of the SFH. The object of this report is to assess the cardiovascular response after hemodilution with SFH having different O_2 affinities. Hemoglobin solutions of different P_{50}'s were obtained by varying the yield of covalently linked pyridoxalated hemoglobin (SFH-P) in the SFH solution. Twelve awake, paralyzed baboons had normovolemic exchange transfusions to hematocrits below 6. The table lists the means ± 1 SEM of the response to hemoglobin solutions with different P_{50}'s. We show the whole blood P_{50} (corrected to the animal's pH, pCO_2, and temperature), hematocrit (Hct), whole blood hemoglobin concentration(hb), cardiac output (CO), heart rate (HR), stroke volume (SV), mean arterial pressure (MAP), and mean pulmonary arterial pressure (MPAP). The first row of the table contains the data from the control period.

P_{50} (mmHg)	Hct (%)	Hb (gm%)	CO (L/m)	HR (bmp)	SV (cc)	MAP (mmHg)	MPAP (mmH
30.8+0.9	35.1+1.3	11.7+0.4	3.2+0.2	108+4	30.6+2.7	126+4	13.9+2.
22.6±0.5	2.8 ±.7	5.5±.4	3.0±.3	119±4	25.6±2.7	91+10	19.2±4.
19.9±0.4	4.4+1.1	5.5±.3	2.8±.3	113±12	22.8±4.3	98±12	21.+5.1
16.7±0.2	3.5±.7	5.2±.6	2.5±.4	126±11	22.±4.4	109±8	17.6±6.
13.4±0.4	2.6±.9	4.8 ±.3	2.6±.4	139±10	19.2±3.8	98±7	16.3±5.

We conclude that cardiac output is unaffected by changes in whole blood P_{50}, at a given level of hemodilution with SFH.

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7. HEMODYNAMIC RESPONSE IN THE BABOON TO EXTREME HEMODILUTION USING STROMA-FREE HEMOGLOBIN

We have shown previously that normal-volemic (NV) exchange transfusion (ET) with Dextran-70 produces a linear increase in cardiac output (CO), while a similar ET with a stroma-free hemoglobin (SFH) produces no such change in the awake baboon. Since the lowest hemoglobin concentration ([Hb]) in the hemoglobin exchange group was 6 gms %, it is not clear whether the unchanged CO was related to insufficient anemic stimulation. Thirteen baboons had total NV ET with SFH to reduce their final [Hb] to 4 gms. %. The table lists the mean ± 1 SEM of the response to declining [Hb]. We show the hematocrit (Hct), heart rate (HR), stroke volume (SV), cardiac output (CO), mean arterial pressure (MAP) and mean pulmonary arterial pressure (MPAP).

Hb (gms.%)	Hct (%)	HR (bpm)	SV (cc)	CO (L/min)	MAP (mmHg)	MPAP (mmHg)
11.7±.4	34.9±1.2	107±4	31±2	3.2±.2	130±4	14±2
5.7±.02	5.8±1.6	124±6	22±3	2.7±.3	122±8	17±5
5.0±.1	2.8±.6	135±4	19±1	2.5±.1	87±3	17±2
4.2±.1	1.5±.3	132±5	22±2	2.8±.2	94±6	16±2

It is concluded that the total ET with SFH to extreme anemia ([Hb] = 4) does not stimulate an increase in CO.

8. A QUANTITATIVE ANALYSIS OF ALTERATIONS IN $P\bar{v}O_2$

The mixed venous oxygen tension ($P\bar{v}O_2$) is an indication of mean tissue pO_2. Although frequently measured, the etiology of an abnormal value is often unclear. The five determinants of $P\bar{v}O_2$ are: cardiac output (C.O)., arterial saturation (Sa), hemoglobin concentration ([Hb]), oxygen affinity (P_{50}), and oxygen consumption $\dot{V}O_2$. Although an isolated change in any parameter can result in an altered $P\bar{v}O_2$, simultaneous changes in these variables are more common in the clinical setting. The purpose of this report is to describe a quantitative

method of assessing the influence of these factors on $P\bar{v}O_2$ during the induction of acute normovolemic anemia.

Data from nine unanesthetized baboons undergoing exchange transfusion with Dextran-75 was analyzed retrospectively. During the study the average [Hb] fell from 12.9 to 2 gm%; the C.O. increased from 3.2 to 4.6 L/min; and the $P\bar{v}O_2$ decreased from 47.5 torr to 27 torr. Small decreases were observed in $\dot{V}O_2$ and P_{50}, and the Sa did not change.

A multiple linear equation was developed which calculated $P\bar{v}O_2$ as a function of $\dot{V}O_2$, [Hb], C.O. and P_{50}. The resulting equation, based on 36 sets of observations, was:

$$P\bar{v}O_2 = 24.2 + 2.3\ [Hb] + 2.0\ C.O. - 1.7\ \dot{V}O_2 - 0.076\ P_{50}$$

The S.E. of estimate was 5 torr and the correlation coefficient (R) was 0.85 ($p<.001$). This indicates that an increase in [Hb] of 1 gm% will raise the $P\bar{v}O_2$ by 2.3 torr, if other factors remain constant. An increase in C.O. of 1 L/min will raise the $P\bar{v}O_2$ by 2.0 torr.

CONCLUSION: This multiple linear regression equation permits a quantitative description of the factors that influence $P\bar{v}O_2$, and may be useful in predicting the effect of changes in these determinants on tissue oxygenation.

9. THE CONTRIBUTION OF HEMOGLOBIN CONCENTRATION AND P_{50} TO OXYGEN TRANSPORT DURING EXCHANGE TRANSFUSION WITH HEMOGLOBIN SOLUTIONS.

The ability of a hemoglobin solution to maintain oxygen transport is dependent upon hemoglobin concentration ([Hb]) and hemoglobin-oxygen affinity (P_{50}). We previously have shown that the effect of increasing P_{50} was a significant increase in the mixed venous tension ($P\bar{v}O_2$). Alterations in [Hb] also will influence $P\bar{v}O_2$. The purpose of this report is to study the

quantitative relationships between [Hb], P_{50}, and $P\bar{v}O_2$ during exchange transfusion (ET) with stroma-free hemoglobin solution (SFH).

Fifteen awake, paralyzed baboons were exchange transfused randomly with SFH or with pyridoxalated SFH. Oxygen consumption ($\dot{V}O_2$), cardiac output (C.O.), [Hb], P_{50}, $P_{a_{O2}}$, and $P\bar{v}O_2$ were obtained from baseline to 0 hematocrit. Since $P\bar{v}O_2$ is a function of C.O., [Hb], P_{50}, and $\dot{V}O_2$, a multiple linear regression model was used to assess the relative contribution of each of these determinants to the observed $P\bar{v}O_2$.

Seventy-seven sets of observations were obtained. Significant decreases occurred in [Hb] (12 to 4 gm%), P_{50} (35 to 18 mmHg), and $P\bar{v}O_2$ (50 to 17 mmHg), while $\dot{V}O_2$ and C.O. only showed random variations. $P\bar{v}O_2$ can be described by the regression equation:

$$P\bar{v}O_2 = 5.7\ C.O. + 3.9\ [Hb] + 0.45\ P_{50} - 4.2\ \dot{V}O_2 - 6.8$$

The correlation coefficient (R) was 0.95 with standard error of estimate = 4 mmHg. The contribution of each parameter therefore can be assessed by the magnitude of its coefficient in the equation. This indicates that an increase in [Hb] of 1 gm% or a P_{50} shift of 9 mmHg, will raise the $P\bar{v}O_2$ by 3.9 mmHg.

Our data suggests that tissue hypoxia during ET with SFH may be avoided more easily by raising the [Hb] than by altering P_{50}.